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Research Brief

Sarcocystis neurona: Molecular characterization of enolase domain I region and a comparison to other protozoa

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ABSTRACT

Sarcocystis neurona causes protozoal myeloencephalitis and has the ability to infect a wide host range in contrast to other *Sarcocystis* species. In the current study, five *S. neurona* isolates from a variety of sources, three *Sarcocystis falcatula*, one *Sarcocystis dasypi/S. neurona*-like isolate, and one *Besnoitia darlingi* isolate were used to compare the enolase 2 gene segment containing the domain I region to previously sequenced enolase genes from *Neospora caninum*, *Neospora hughesi*, *Toxoplasma gondii*, *Plasmodium falciparum*, and *Trypanosoma cruzi*; enolase 2 segment containing domain I region is highly conserved amongst these parasites of veterinary and medical importance. Immunohistochemistry results indicates reactivity of *T. gondii* enolase 1 and 2 antibodies to *S. neurona* merozoites and metrocytes, but no reactivity of anti-enolase 1 to the *S. neurona* bradyzoite stage despite reactivity to *T. gondii* bradyzoites, suggesting expression differences between organisms.

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1. Introduction

Sarcocystis neurona is an apicomplexan parasite and the most common etiologic agent of equine protozoal myeloencephalitis (EPM), one of the most frequently diagnosed neurologic diseases of horses in North America (Dubey et al., 2001). The parasite is unique within the *Sarcocystis* genus in its ability to infect a wide host range (Dubey et al., 2001). In this regard, *S. neurona* is like *Toxoplasma gondii* and *Neospora caninum* which are both within the Sarcocystidae family and are able to infect a wide range of intermediate hosts. Yet, recrudescence of *S. neurona* infection has yet to be shown. Therefore, characterization of stage-specific proteins may provide helpful biological information regarding stage transformation events, metabolic requirements and when comparing isolates and parasite life stages from different hosts. Recently, antigenic diversity amongst *S. neurona* strains was recognized for multiple surface antigens (SAG) within the SAG/SRS family of proteins indicating antigenic diversity amongst strains (Marsh et al., 2001; Howe et al., 2007). These results indicate that stage-specific family of proteins would be parasite strain dependent and associated reagents developed may not be completely useful for examining different isolates representing the same life cycle stage.

Enolases from a wide variety of organisms have been characterized and their sequences are highly conserved (Pancholi, 2001). Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is an enzyme responsible for catalyzing the only dehydration step in the glycolytic pathway (Van der Straeten et al., 1991). Specifically, the expression of enolase 1 and 2 genes has been linked to stage conversion of *T. gondii*. (Dzierszinski et al., 2001). *Toxoplasma gondii's* expression of enolase 1 enzyme may be related to its reliance on anaerobic glycolysis during encystment. Bradyzoite formation in *T. gondii* is promoted through the inhibition of both the host cell's and parasite's mitochondria (Gross et al., 1996). In contrast, the actively dividing and invasive tachyzoite expresses enolase 2 and not enolase 1. Therefore, it has been proposed that differential gene expression in protozoan parasites probably serves as a strategy for these parasites to adapt to different environmental conditions (Kibe et al., 2005).

However, enolase is not only associated with a parasite's metabolic pathway. Other studies have characterized and shown potentially other roles for this enzyme in different protozoa. Enolase proteins are found in *Plasmodium falciparium* (Pal-Bhowmick et al., 2007a), *Leishmania donovani* (Gupta et al., 2007), *Leishmania mexicana* (Quiñones et al., 2007), and *Eimeria tenella* (Labbé et al., 2006). *Leishmania mexicana* enolase can bind host plasminogen as shown *in vitro* (Vanegas et al., 2007). *Plasmodium* spp. enolase is immunostimulatory (Pal-Bhowmick et al., 2006), and anti-*Plasmodium* enolase antibodies can be protective (Pal-Bhowmick et al., 2007b). These studies further support

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enolase as an exploitable target molecule for immunoprophylaxis of parasite stages. Interestingly, investigators discovered three separate enolases for *Trypanosoma cruzi*, two are highly similar and a third is a distinctly different (Keeling and Palmer, 2000; El-Sayed et al., 2005). Cross-reactivity of individual enolase isoforms between related parasites has been described. For example, anti-*T. gondii* enolase 2 antibody demonstrated cross-reactivity to *Neospora* spp. tachyzoites and *S. neurona* merozoites by immunoblot analysis, but the anti-*T. gondii* enolase 1 antibody did not react with *S. neurona* merozites by immunoblot analysis (Wilson et al., 2004). Wilson et al. did not evaluate tissue cyst stages of *S. neurona* to determine the reactivity of anti-*T. gondii* enolase 1 to *S. neurona* bradyzoites as it was reported with *T. gondii* bradyzoites (Ferguson et al., 2002). However, based on the sequence homology of known DNA sequences of enolase 1 and 2 between Sarcocystidae parasites, it would be expected that the proteins may be very similar and may show similar expression profiles.

The focus of this study was to determine the enolase 2 sequence conservation from multiple *S. neurona* isolates obtained from different hosts and to compare the predicted amino acid sequence to other Sarcocystidae. Immunoblot analysis of multiple *Sarcocystis in vitro*-derived merozoites was evaluated using anti-enolase 2 antibody. In addition, the study evaluated parasite stage expression of enolase 1 and 2 in host tissues containing *S. neurona* merozoites, schizonts, and tissue cysts containing metrocytes and bradyzoites in comparison to the equivalent acute or chronic stages of *T. gondii*.

2. Materials and methods

Sarcocystis neurona isolates SN-UCD1 (Marsh et al., 1996), SN-37R (Sofaly et al., 2002), SN-MU1 (Marsh et al., 2001), SN-MU2 (A. Marsh, unpublished), SN-MUCAT2 (Turay et al., 2002), and SN-OT1 (Lindsay and Dubey, 2001), and *Sarcocystis falcatula* isolates SF-Cornell, SF-Daft, and SF-Florida (Marsh et al., 1999) were grown in equine dermal cells (ED; American Type Culture Collection, CCL57) as previously described by Wilson et al. (2004). Uninfected ED cells were used as negative control material for contamination of host cells in parasite preparations.

Sarcocystis sp. originally from an armadillo intermediate host was established *in vitro* using natural and laboratory hosts. Briefly, tongue of an armadillo from Florida containing sarcocysts was fed to a laboratory-raised opossum that shed sporocysts; the sporocysts were fed to interferon gamma gene knock out (KO) mice that subsequently developed encephalitis with demonstrable schizonts and merozoites (Cheadle et al., 2002). Merozoites from the brain of a KO infected mouse were used to establish cultures. Within the study, this parasite is referred to as *Sarcocystis dasypi/S. neurona*-like and is designated SD-Fl1. *Besnoitia darlingi* (Bd-Mo) was obtained from a naturally infected opossum in Missouri. Tissue cysts were dissected from the liver surface and were frozen. Infected liver tissue was processed for histologic evaluation. Frozen *B. darlingi* tissue cysts were used for DNA extraction. The *T. gondii* and *Neospora* spp. genomic DNA (gDNA) was kindly provided by Wilson et al. (2004).

The gDNA of parasite and host cells were isolated by the use of DNeasy tissue kit (Qiagen, Valencia, CA, USA). PCR reactions using MALDI-F (5′-GCG TCT ACG GGT ATT TAT GAG G-3′) and MALDI-R (5′-GAA AAA CGG TAC AGG CAT AAC CAT C-3′) primers were performed, analyzed and purified for sequencing as described (Wilson et al., 2004). Each of the purified PCR products was added to 6 pmol of either forward or reverse MALDI primers separately, and complete double-stranded nucleotide sequences were obtained with the automated 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and BigDye Terminator Cycle Sequencing. Final DNA sequence construction, alignments, and comparisons were facilitated using Vector NTI (Invitrogen, Carlsbad, CA, USA).

To evaluate protein expression of enolase 1 and 2 in *S. neurona*, immunohistochemical staining (IHC) was performed using the Dako Envision Plus System (DAKO Corporation, Carpinteria, CA, USA). Antibodies specific to *T. gondii* enolase 1 and 2 (Ferguson et al., 2002) were used and titrated over a series of dilutions from 1:500 to 1:5000 against both *S. neurona* and *T. gondii* infected tissues from acutely and chronically experimentally infected animals. Mice were used for *T. gondii* acute (tachyzoite) and chronic (bradyzoite) infections (Dubey et al., 1995); mice were also used for *S. neurona* acute (merozoite and schizont) infection (Dubey et al., 2000), and raccoon diaphragm was used to obtain the *S. neurona* tissue cyst stage (SN-37R). In addition, tissues from a naturally infected equine were used since experimentally infected animal tissues for SNMU2 isolate were not available. *Sarcocystis neurona* isolates used in IHC included: SN-UCD1, SNMU2, SN-MUCAT2, and SN-37R.

Immunoblot analysis of the *S. neurona* isolates, *S. falcatula* isolates, *T. gondii*, and host cells were performed as previously described (Wilson et al., 2004). Only, culture-derived merozoites and tachyzoites were used for immunoblot analysis as purified preparations of *S. neurona* bradyzoites without contaminating metrocytes were not available.

3. Results and discussion

The DNA obtained from the PCR of *S. neurona*, *S. falcatula, S. dasypi/S. neurona*-like, and *B. darlingi* isolates each produced a single amplification product of approximately 300 bp. The maximum length of sequencing results was 290 bp. No product was present with ED cell DNA. Nucleotide (nt) sequences submitted to GenBank were assigned Accession Nos. as follows: SN-37R (AY660720), SN-MU2 (AY660721), SN-MUCAT2 (AY660722), SN-MU1 (AY660723), SN-OT1 (AY660724), SF-Florida (AY660725), SF-Cornell (AY730639), SF-Daft (AY730640), SD-Fl1 (AY874059), and *B. darlingi* (AY730641). The DNA sequences obtained from all *S. neurona/S. neurona*-like isolates were the same. The sequences of *S. falcatula* isolates had 1 bp difference at nt 157, except SF-Daft, whose sequence was identical to that of the *S. neurona* isolates. This nucleotide change had no effect on the predicted amino acid sequence. The PCR assay and sequencing reaction did not target the entire enolase 2 gene, but enough of the DNA sequence was acquired to compare domain I and differentiate between the *Sarcocystis* spp. and *B. darlingi* DNA. *Sarcocystis neurona* DNA sequence showed 82% similarity to *B. darlingi*, 79% similarity to *T. gondii* (AY155668), 62% similarity *Plasmodium falciparum* (XM001347404), and 54% similarity to *T. cruzi* (AF159531). Only the most similar enolase genes from each of the representative parasites were compared. When the predicted amino acid sequences from the DNA sequences were compared, there were no changes between the plant domain in *S. neurona*, *S. dasypi/S. neurona*-like, and *S. falcatula,* and there was only 1 amino acid difference in this domain in *B. darlingi* and none when compared with the published *T. gondii* sequence (Fig. 1).

For the IHC staining, the anti-enolase 1 antibody at 1:5000 only reacted with *S. neurona* merozoites and schizont stages (Fig. 2A1), and did not react with any zoites within the tissue cyst (Fig. 2B1). The anti-enolase 2 antibody used at 1:5000 dilution showed strong reactivity to *S. neurona* merozoites and schizonts (Fig. 2A2). There was no difference in merozoite or schizont staining results between the isolates of *S. neurona*. In addition, the anti-enolase 2 antibody showed discrete and mostly peripheral staining to individual zoites, presumably metrocytes, within the *S. neurona* tissue cyst without staining the cyst wall or complete

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S. neurona[a]   (ENO2) 1 ENIHKIIKPALIGKDPCDQKGIDKLMVEELDGTKNEWGWCKSKLGANAILAVSMACCRAGAAAKGLPLYKYIATLAGKPTDKMVMPVPFF
S. dasypi[a]    (ENO2) 1 ..........................................................................................
S. falcatula[a] (ENO2) 1 ..........................................................................................
B. darlingi[a]  (ENO2) 1 .....................................L.....................M...........N............
T. gondii[b]    (ENO2) 1 .....................................................M...........N............

T. gondii[b]    (ENO1) 1 .IVRQE.....L...........M....Q.........YS..........G..I........S..............TI..........
N. caninum[c]   (ENO1) 1 .IVQ.E...Y.L...........M....Q.........YS..........GI.I.......................I..........
N. hughesi[d]   (ENO1) 1 .IVQ.E...S.L...........M....Q.........YS..........GI.I.......................I..........
P. falciparum[e]       1 K..NE..A.K...MNCTE..K..N........S......S...........I...V.......NKVS....L.Q....KS.Q..L...CL
T. cruzi[f]            1 K.VNDVLA...V...ELQ.STL....-RD....P.-----..........GC...ISK.A..R..V...R.L.E...--.KEVRL...C.
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[a]Result determined within this study.
[b]GenBank sequence AY155668.
[c]GenBank sequence AY563178.
[d]GenBank sequence AY563179.
[e]GenBank sequence XM_001347404, designation of enolase 1 or 2 not provided in the cited GenBank accession.
[f]There are at least three isoforms of enolase reported for *T. cruzi*, with two (EAN93876; EAN81679) significantly different than GenBank EAN97849 which is shown in the alignment

Fig. 1. Comparative the partial predicted amino acid sequence data of putative enolase 2 gene (ENO2) from a representative sequence of *Sarcocystis falcatula* (AY730639) and sequences of *Sarcocystis dasypi/S. neurona*-like (AY874059), and *Besnoitia darlingi* (AY730641), aligned with predicted amino acid sequences from *Toxoplasma gondii* ENO2 (AY155668) and enolase 1 (ENO1) (AY155668), *Neospora caninum* ENO1 (AY563178), *Neospora hughesi* ENO1 (AY563179), *Plasmodium falciparum* enolase (XM_001347404), *Trypanosoma cruzi* ENO 1 (AF159530) and ENO2 (AF159531), all in reference to an ENO2 representative sequence of *Sarcocystis neurona* (AY563177). The underlined region represents the plant motif, EWGWC. Dashes represent no amino acid residue but are inserted for alignment purposes while dots indicate conserved predicted amino acids.

cyst containing bradyzoites (Fig. 2B2 and B2a). Non-specific background staining of host tissue occurred at the lower dilutions. At the higher dilutions, the background staining was lost, and there was little difference in parasite reactivity between the 1:2500 and 1:5000 dilutions of the antibody. The *T. gondii* control stages stained as previously reported (Ferguson et al., 2002). Briefly, the anti-enolase 1 antibody reacted only with *T. gondii* bradyzoites, and the anti-enolase 2 antibody reacted only with *T. gondii* tachyzoites (Fig. 2C1 and C2). No cross-stage staining was evident in *T. gondii* at the higher dilutions (1:2500, 1:5000).

Immunoblot analysis, using the anti-enolase 2 antibody, showed similar results as previously reported (Wilson et al., 2004). The parasite preparations showed a reactive band at approximately 47 kDa, and the host cell preparation indicated a relatively weak band at approximately 49 kDa (Fig. 3).

Apicomplexan enolase is a protein thought to be derived from photosynthetic lineage by a secondary endosymbiosis event between green algae and apicomplexans and has been described in a number of other parasites (Hannaert et al., 2000; Keeling and Palmer, 2001). Previous studies have established two distinct forms of enolase, enolase 1 and enolase 2, in *T. gondii* (Dzierszinski et al., 2001; Ferguson et al., 2002). Enolase 1 is expressed primarily in the bradyzoites of *T. gondii*, while enolase 2 is more highly expressed in tachyzoites (Dzierszinski et al., 2001; Ferguson et al., 2002). When the tachyzoite to bradyzoite conversion occurs in *T. gondii*, the enolase 1 gene is either over-expressed or exclusively

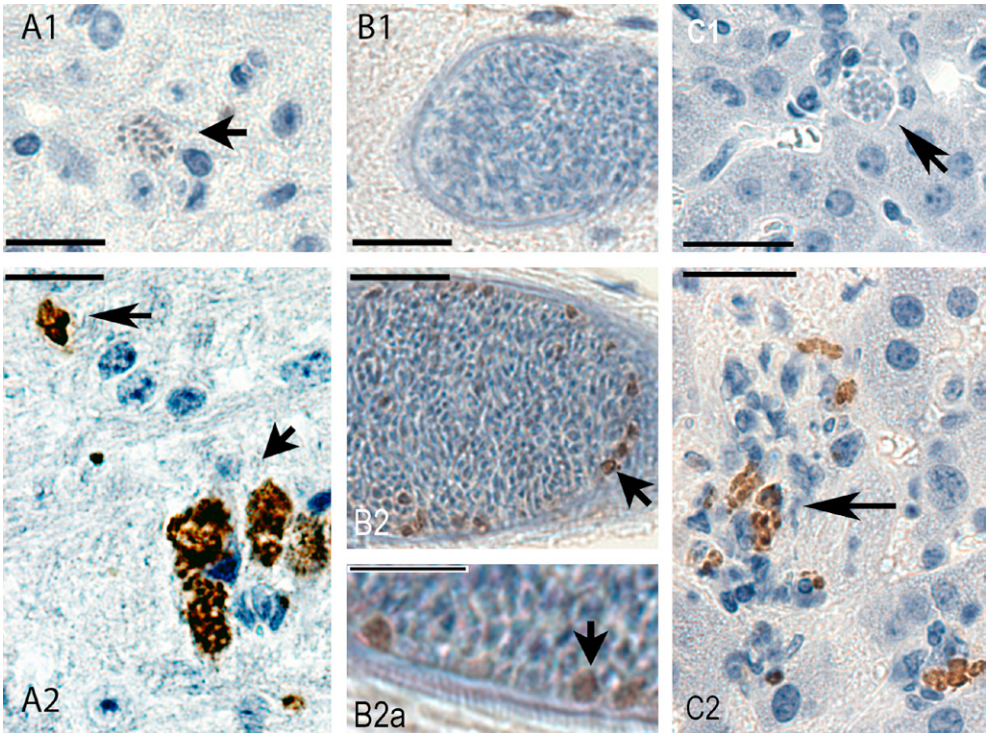

Fig. 2. Immunohistochemical staining of histologic sections of tissues from acute and chronically experimentally infected *S. neurona* animals, SN-37R, and *T. gondii* control tissues. A1–C1 were stained with anti-enolase 1 antibody; A2–C2 were stained with anti-enolase 2 antibody. Anti-enolase 1 antibody reacts weakly to *S. neurona* merozoites (A1, arrows) in mouse brain, but shows no reactivity to *T. gondii* tachyzoite controls (C1); in contrast, anti-enolase 2 antibody reacts strongly to schizonts and merozoites (A2, arrows) in mouse brain and *T. gondii* tachyzoites (C2, arrows) (bar is 20 μm). Anti-enolase 1 antibody shows no reactivity to the tissue cyst in raccoon muscle (B1), but anti-enolase 2 reacts with putative metrocytes (B2 and B2a, arrows) inside the cyst (bar is 20 μm). With higher magnification, reactivity of individual metrocyte (B2a, arrow) with anti-enolase 2 antibody is seen but no reactivity is seen with adjacent bradyzoites (scale bar is 10 μm).

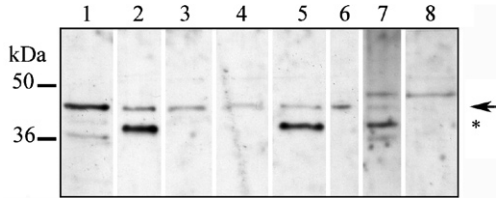

Fig. 3. Protein immunoblot probed with anti-enolase 2 antibody. Proteins loaded were as follows: lane 1, *Sarcocystis neurona*, SN-UCD1; lane 2, *S. neurona*, SN-37R; lane 3, *S. neurona*, SN-OT1, *S. neurona*; lane 4, SN-MUCAT2; lane 5, *S. falcatula*, SF-Florida; lane 6, *S. falcatula*, SF-UCD1; lane 7, *T. gondii*, RH; and lane 8, equine dermal cells. Parasite enolase is distinguishable from the host cells as a smaller molecular sized protein (arrow). Smaller molecular reactive sized proteins (∗) suggest either degradation of the parasite protein lysate or an additional isoform of smaller molecular weight in addition to the approximately 47 kDa protein. Molecular masses are given on the left in KDa.

expressed at both transcriptional and protein levels within the cyst stage (Kibe et al., 2005). These two enzymes share amino acid similarity but are still antigenically distinct (Dzierszinski et al., 2001; Ferguson et al., 2002), and transcriptional gene regulation for *T. gondii* has recently been reported (Kibe et al., 2005).

Enolase has also been shown to be a significant enzyme in the glycolytic pathway of another apicomplexan, *Plasmodium falciparum*, and it contains the pentapeptide, EWGWS insertion (Read et al., 1994). This plant motif pentapeptide or domain I contributes to enolase enzymatic activities (Dzierszinski et al., 2001). *Neospora hughesi* and *N. caninum* enolase predicted amino acid sequence indicate the presence of a similar plant-like motif, EWGYS, (Wilson et al., 2004) previously identified in *T. gondii* enolase 1 (Dzierszinski et al., 2001). The *S. neurona*, *S. dasypi/S. neurona*-like, and *S. falcatula* predicted enolase protein sequence in this study contains the true plant motif EWGWC characteristic of enolase 2 and identified in *T. gondii* enolase 2. Thus, the sequencing results in this study suggest the identification of a putative enolase 2 gene of *S. neurona*.

The high conservation of enolase 2 domain I region suggests this feature can be exploited for comparative studies amongst a wide range of protozoa that undergo stage transformation and associated metabolic changes. Since DNA sequence and predicted amino acid sequence similarity between the *Sarcocystis* spp., *B. darlingi*, and *T. gondii* enolase 2 evaluated show few changes the protein may be highly conserved and serve similar functions in these parasites. Performing a more extensive study of Sarcocystidae may be of interest to determine the complete range of conservation of enolase 2 within this family of parasites. *Sarcocystis neurona* is unique because of its ability to infect multiple intermediate hosts. The intermediate host range diversity for *S. neurona* is atypical when compared to other *Sarcocystis* spp. or *Besnoita* spp. which are fairly host specific. Results from an *S. neurona* expressed sequence tag (EST) project using the *S. neurona* isolate cDNA library, UK cSN-4, have produced sequences identified as enolase (examples, CV194305 and CV193162) that have expanded the sequence region characterized in this study. However, not all the sequences available in the EST project are not without nucleotide ambiguities. As of yet, the complete *S. neurona* enolase 2 gene has not been described.

Interestingly, the expression levels of enolase 1 and 2 determined for *S. neurona* in the host tissues were not completely identical to the *T. gondii* stages evaluated. *Sarcocystis neurona* merozoites and schizonts reacted with both anti-enolase 1 and 2 antibodies. The staining for anti-enolase 1 was much weaker as compared to the very strong staining of anti-enolase 2 to the *S. neurona* merozoites and schizonts. The *S. neurona* bradyzoites did not react with the anti-enolase 1 antibody in contrast to the *S. neurona* metrocytes' and *T. gondii* bradyzoites' strong reactivity with the anti-enolase 1 antibody. These results suggest the existence of at least two putative enolase proteins in *S. neurona*. This parasite stage-specific staining suggests differential expression of these proteins in *S. neurona* indicating previously unrecognized protein activity between the metrocyte stage of *S. neurona* and *T. gondii* bradyzoite, suggesting a shared epitope recognized by the anti-enolase 1 antibody.

Even though the antibodies used in this study were produced against *T. gondii* enolase 1 and 2 and not *S. neurona* enolase, they successfully demonstrated unique antigens associated with metabolic pathways present between merozoites, metrocytes and bradyzoites of *S. neurona*. Nevertheless, these results suggest additional studies need to be pursued to address the differential enolase staining observed since it is not exactly the same as seen with *T. gondii*. The results suggest the *S. neurona* metrocytes in the cysts express enolase 2 but then stop after differentiating to the bradzyoite stage, indicating metabolic and protein expression differences and requirements between these two *S. neurona* stages present inside the tissue cyst. A better understanding of parasite metabolic processes, active replication and encystment process provides opportunities for immunoprophylaxis intervention.

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